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LINKAGE IN PEROMYSCUS, AND SEQUENTIAL TESTS FOR INDEPENDENT ASSORTMENT

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INTRODUCTION

THE results of eight linkage tests in Peromyscus maniculatus made at the Laboratory of Vertebrate Biology are reported. Attention is called to the use of sequential analysis for making these tests. Sequential analysis was developed by Abraham Wald and his co-workers (Wald, 1947) during World War II and has since been widely employed as a sampling technique in preference to fixed sample size methods. Sequential analysis allows statements of predetermined precision to be made about the results of a linkage test with the smallest possible sample size.

Acknowledgments

Some of the linkage tests reported here were commenced by Elizabeth Barto. Classification of waltzing mice was made mostly by Arthur E. Dammann and Jean C. Eggleton. Popie Mohring made some brown-tip classifications microscopically. Donald G. Vernall conducted three of the tests and aided materially in other ways. I am indebted to William J. Schull for originally suggesting that sequential analysis be used for these tests, and to Drs. Barto, Schull, and Newton E. Morton for much stimulating discussion and for their critical reading of the manuscript. Two linkage tests previously reported were repeated at the suggestion of Dr. Lee R. Dice, who supported and encouraged the entire program. Grace Yesley prepared the figures. This investigation was supported in part by research grant M-375 from the National Institutes of Health, Public Health Service.

SEQUENTIAL ANALYSIS

In a linkage test the primary interest lies in determining whether the gametic recombinant fraction, Θ, is Θ_0 ($\Theta_0 = .50$ if no linkage exists) or Θ_1, $0 \leq \Theta_1 < \Theta_0$. In the present paper the possibility that $\Theta > .50$ is ignored. It is desirable that, whichever alternative is accepted, the probability of an erroneous choice being made is so small that the adverse consequences of a wrong decision need not be contended with very frequently.

It has long been customary in linkage tests to measure linkage by the proportion of recombinant gametes. This custom has been adhered to in the present paper, and it involves a rearrangement of the formulas usually found in the literature dealing with sequential tests. The notation and formulas here generally follow those of Wallis (1947), who gives a concise and useful summary of sequential analysis.

In general, the sample proportion, p, of items which are of the recombinant phenotype is not the same as the proportion of recombinant gametes. Hence it is necessary to use in the analysis the observed proportion of phenotypically recombinant individuals and to relate this proportion to the gemetic recombinant fraction by consideration of the mating system in use. Only in the case of a double backcross (testcross) is equality expected between the proportion of recombinant phenotypes and the proportion of recombinant gametes produced by the parents. In addition to Θ and the mating system, p depends upon single factor segregation and upon sampling error. It is the latter factor which makes use of a sampling procedure mandatory.

In practice, therefore, it must be determined whether p is p_0 or p_1. Intuitively, in a test made to ascertain whether linkage exists

p_0 should be the value expected in the event that $\Theta = .50$, including all cases in which the genes are borne on different chromosomes. The factor p_1, then, should be some value which indicates that linkage does exist between the loci under test. It is also desired that the probability of stating incorrectly that linkage does exist (a type I error) should be no larger than α. It is equally desirable that if linkage as tight as, or tighter than, the amount indicated by p_1 exists, the probability of overlooking the fact (a type II error) be no larger than β. Finally, when linkage does exist, it generally will be desirable not only to detect the existence but also to estimate the degree of linkage.

The sequential test, therefore, requires prior specification of the factors, α, β, p_0, and p_1. The values assigned to α and β depend principally upon the consequences of a wrong decision. The relationships between p_0 and p_1 depend upon the particular problem. For the present purpose p_0 must be the larger. The difference may be whatever amount best serves the purpose. Numerical specification of these four factors is discussed later. The fifth factor involved in a sequential analysis, sample size, N, is treated as a variable.

To perform a sequential linkage test, after α, β, p_0, and p_1 have been selected, certain operating factors must be determined. First compute

$$a = \log \frac{1 - \beta}{\alpha}\text{, and} \tag{1}$$

$$b = \log \frac{1 - \alpha}{\beta}. \tag{2}$$

From these, compute

$$R_0 = \frac{b + N \log \left(\frac{1 - p_1}{1 - p_0}\right)}{\log \left[\frac{p_0}{p_1}\frac{(1 - p_1)}{(1 - p_0)}\right]}\text{, and} \tag{3}$$

$$R_1 = \frac{- a + N \log \left(\frac{1 - p_1}{1 - p_0}\right)}{\log \left[\frac{p_0}{p_1}\frac{(1 - p_1)}{(1 - p_0)}\right]}. \tag{4}$$

Common logarithms may be used for these equations. Another computation, the average sample size required to accept the null hypothesis if the null hypothesis is true, is not needed for the analysis but is used in later discussion. The average sample size, $E[N_0]$, is

$$E[N_0] = \frac{b - \alpha(a + b)}{\log\left[\left(\frac{p_0}{p_1}\right)^{p_0}\left(\frac{1 - p_0}{1 - p_1}\right)^{(1 - p_0)}\right]}. \tag{5}$$

Equations (3) and (4) describe a pair of parallel lines, line R_0 with a positive intercept and line R_1 with a negative intercept. These are called statistical decision lines and should be drawn on a graph with N as the abscissa and R as the ordinate. The analysis is now done graphically. For each increment of data plot on the graph the cumulative number of recombinants, R, against the cumulative sample size, N. If the last point ever lies on or above the R_0 line, the experiment may be terminated and the null hypothesis, $p = p_0$, accepted. While the last point lies between the decision lines, collection of data must continue, as neither hypothesis can be accepted without exceeding one of the predetermined error levels. If the last point lies on or below the R_1 line, the alternative hypothesis, $p = p_1$, is accepted. When the alternative hypothesis is accepted, it usually will be desirable to continue collection of data in order to estimate more precisely the magnitude of Θ. The use of sequential analysis for this estimation has not been satisfactorily worked out (see Wald, 1947, Chap. 11), and hence the estimation will be done in the conventional manner.

The use of graphs in the analysis is illustrated in Figures 2-7, which give the results of the six tests for which no linkage was discovered. The constants necessary for drawing the decision lines are determined later. Figures 2-6 illustrate testcross matings and Figure 7 illustrates the only F_2 test made, in this case a repulsion F_2. Only the terminal part of each analysis is shown, in order to increase the relative size of the critical part of the analysis in the figures.

Selection of the constants may next be made. Factor p_0 is easiest to specify, since in the event that no linkage exists, p_0 can take only one value for any mating system, in the absence of poor single gene segregation. For linkage tests in peromyscus, most species of which have 23 pairs of autosomes (Cross, 1936; Makino, 1953), the majority of linkage tests will fail to demonstrate linkage. Thus we have specified p_0 to be the proportion of recombinants expected if $\Theta = .50$. This, for example, gives $p_0 = .50$ for a testcross and $p_0 = .625$ for a repulsion F_2 generation.

In a test made to ascertain the linear order of genes already known to be linked, other possibilities for p_0 exist. Suppose, for example, that locus A is known to be linked to locus B with 18 per cent crossing over, and locus C is also known to be linked to locus B with 13 per cent crossing over. Then loci A and C should be linked with either (approximately) 31 per cent or 5 per cent crossing over. For this case p_0 would be .31 and p_1 would be .05 for a testcross, and there would be appropriate values for other mating types.

Specification of α may be made on general grounds, considering the consequences of making a type I error. In the event that the alternative hypothesis is accepted when the sequential test indicates this choice, it would be very important to select a stringent level for α. In a recent paper dealing with sequential tests for human linkages, Morton (1955) suggested levels ranging from $\alpha = .002$ to $\alpha = .0005$, depending on the power of the test used, and gave a battery of four sequential tests to use for detecting linkage in man. These tests are based on Θ_1 equaling, respectively, .05, .10, .20, and .30, and the test to be used depends chiefly upon the amount of data available to the investigator. In human linkage studies this is the best that can be done in most cases, since the precise estimation of linkage usually requires an impractically large body of data.

For linkage tests in a laboratory animal continued estimation beyond the first point of detection in effect reduces the level of α. In the event that a type I error is made at the termination of the sequential part of the test, continued collection of data for better estimation will in many, perhaps in most, cases indicate that the error had been made. No published report claiming linkage will then appear, and the most serious consequence of making a type I error will be avoided. The only remaining consequence of importance will be the increased amount of effort expended in fruitless estimation.

Furthermore, the plotting of different decision lines for any p's, but for different combinations of α and β, shows that varying α varies the position of the R_1 line considerably and has very little effect on the position of the R_0 line; varying β has the reverse effect. Therefore, since the more probable result of any test is the acceptance of the null hypothesis, a reasonably stringent level for α should not materially increase the sample size when the null hypothesis is true. For these reasons, $\alpha = .01$ has been adopted in these and subsequent linkage tests.

For selection of β the reasoning is different. The true recombinant fraction between most pairs of randomly selected loci is .50, and no amount of data will serve to indicate the loci are linked. It therefore becomes desirable to set some upper limit to the amount of data which will be collected for such cases.

In accepting the null hypothesis one is actually excluding the relation $0 \leq \Theta \leq \Theta_1$. The probability that the stated exclusion is correct is $(1 - \alpha)$ if $\Theta = \Theta_0$. This is equivalent to stating that sequential analysis is here being used to detect independent assortment, where independent assortment is defined as $\Theta_1 < \Theta \leq .50$. Conversely, the probability of claiming independent assortment is $\leq \beta$ if $0 \leq \Theta \leq \Theta_1$. Failure to exclude linkage with some reasonable probability of being correct will most likely lead to repetition of the test by someone. This involves wastage insofar as some animals must be reared which do not contribute linkage information. It thus appears reasonable to plan the experiments so that the probability of overlooking linkage of the specified magnitude is quite low. I have therefore selected $\beta = .01$ to use for these and subsequent linkage tests.

Three of the four necessary factors have now been specified. Once the fourth, p_1, is specified, the average sample size (equation 5) becomes fixed for cases when the null hypothesis is true. In practice, as Morton (1955) also pointed out, average sample size is in reality the limiting factor. Although actual sample size is treated as a variate, it would serve no purpose to assign to the constants values which would necessitate use of an impractically large sample. Hence, at this point, it becomes necessary to specify the order of magnitude of average sample size and to determine from that what values of p_1 are then feasible.

Certain considerations which may be peculiar to this laboratory dictate the choice of average sample size. All the mutant genes which are suitable for linkage tests are carried one to a strain. Most of these genes are recessives. Therefore, two mating types will cover most linkage tests which are likely to be made. If a test is to be made between two recessive genes, no double recessive strain is apt to be available. If it is desired to use a testcross, it is necessary to produce a double recessive strain by crossing the two strains carrying the appropriate mutant genes. This produces a repulsion F_1 generation. From the subsequent F_2 generation, plus matings between selected F_2 animals, a supply of double recessive mice may be obtained, and the testcross may then be made. It may be as efficient, however, from the standpoint of the number of individuals produced simply to obtain the desired information directly from the repulsion F_2 generation. In the event that no linkage is detected, this is all that will be required. If linkage is detected, it will then be necessary to estimate from some other mating type, if it is desired to determine the recombinant fraction for the sexes separately. Detection of independent assortment from a repulsion F_2 generation is the procedure which I have in general adopted, although this, as opposed to the use of a testcross, is a recent development and all but one of the tests reported here were made before that decision.

In the event that a test is desired between a dominant and a recessive mutant, or between two dominants, a double recessive strain is usually already available, and the only mice which must be produced without directly contributing information about linkage are enough double heterozygotes for the testcross matings.

Consideration of the time and space which could on the average be devoted to a linkage test led to the specification of about one thousand animals being reared in any one test, whether this number was to be expended directly or indirectly. I previously had adopted, on rather arbitrary grounds, the condition that about 200 testcross progeny would be reared. Insertion of these numbers into equation (5) for the testcross and the repulsion F_2 generation gives estimates for Θ_1 close to .40 in each instance. Such a value for Θ_1 appeared reasonable and was tentatively accepted. Since some tests may be made at once as testcrosses, with a smaller sample size on the average, others must be made where epistasis exists, as it did in two tests reported here, and these require larger samples than do the nonepistatic cases. Hence, it appeared that over a large series of tests the mean number of mice reared would be in the neighborhood of one thousand.

The same conclusions were reached by a different approach. For any mating which will give linkage information, equation (5) may be solved for N with the previously selected α, β, and p_0, as p_1 assumes different values. The solutions for a testcross and a repulsion F_2 are presented graphically in Figure 1, in terms of sample size and Θ_1. Inspection of the curves shows that, as Θ_1 increases

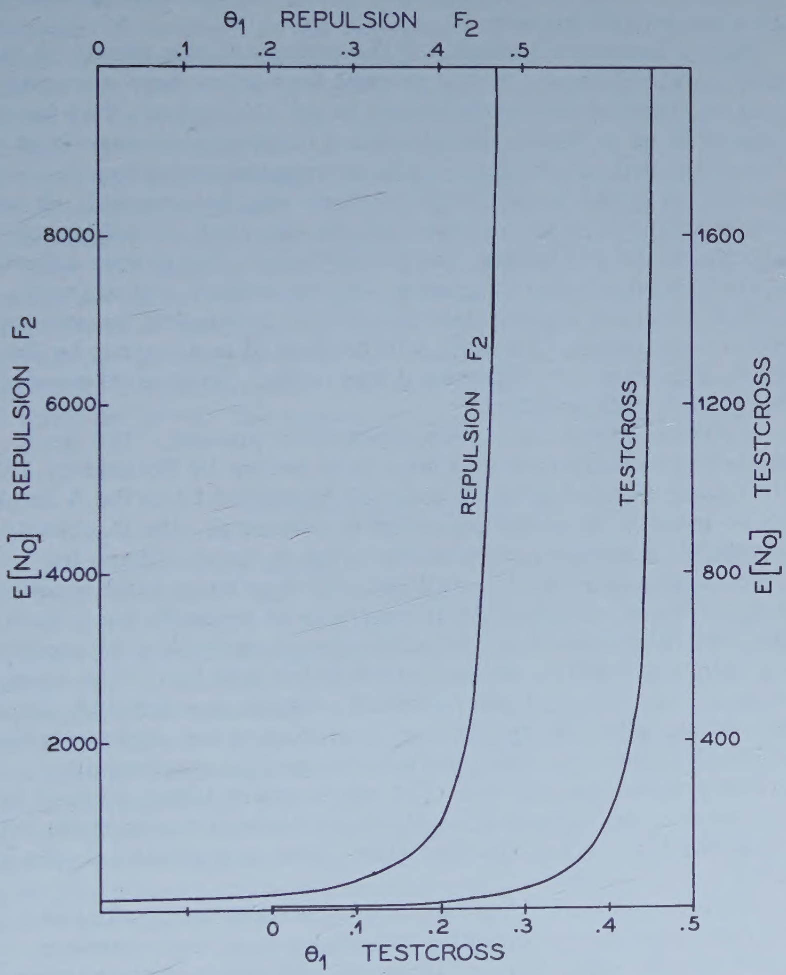

FIG. 1. Average sample number required to accept the null hypothesis, when it is true, as a function of Θ_1. Testcross and repulsion F_2 mating types; $\alpha = \beta = .01$, $\Theta_0 = .50$.

from zero up to about .35, the average sample size is increasing relatively slowly. On the other hand, by the time Θ_1 is in the vicinity of .45, average sample size is increasing extremely rapidly as Θ_1 increases. Clearly somewhere in between these two points should lie the point of diminishing returns. Again, the selection of $\Theta_1 = .40$ appears reasonable.

One remark about the average sampling number is in order. Wallis (1947) stated that the distribution of sample size is skewed so that most samples require fewer than the average number, and that the variance is such that sample size rarely will exceed three times the average sample number.

Before viewing the results of the tests, mention should be made of other considerations. To the present time it has been assumed that the segregation for recombinants is not affected by other factors. This needs to be verified. In the event a conventional analysis of χ^2 indicates that both single factors are segregating properly, the conclusion drawn from the sequential analysis may be accepted. If one or both single factors do not segregate as expected, it will be necessary to adjust the p's to take this into account. The proper adjustment will depend on what is causing the disturbance, for example, differential viability before classification or incomplete penetrance. The final conclusions, however, will be limited in accuracy by the accuracy with which the adjustment was made. No general answer can be given to this problem.

A special case arises when epistasis is present. The word epistasis has had different meanings (see review by Hollander, 1955). The following meaning is used here: If classification at the A locus cannot be made in the presence of the B_i phenotype, the B_i phenotype, but not the B_j phenotype, is epistatic to the A locus. Thus, for example, albino is epistatic to all loci which produce color modifications of the hairs. In tests where epistasis is present, the p's should be assigned values based on the proportion of recombinants expected among individuals which can be classified for both loci. The remaining individuals, consisting of two combined classes, are ignored. In this event, nothing within an experiment conducted in one phase only can distinguish between poor gene segregation and joint segregation disturbance caused by linkage. For single-phase tests, as used in the present report, acceptance or exclusion of linkage can be made only if it is known from other data that single gene segregation occurs as expected.

The effect of collecting data in groups must be considered. As Wald (1947) has shown, grouping can lead only to an increase in sample size, but not to bias, if the groups themselves are random. In the present application two natural groupings larger than the individual occur. The larger is a marking group, which consists of all litters removed from their parents at the same time. Since this operation is performed about once a week in the laboratory, it frequently gives rise to groups consisting of several litters. The marking groups are determined on the basis of the age of the young, not by any information they contribute to a linkage test. The datum points plotted in Figures 2-7 represent marking groups.

The other group is the litter. The order in which litters are removed from their parents is random with respect to the information the litter has to contribute, and hence the litter may be used as a group in the order in which the litters were marked.

The individual is the smallest possible unit which can be used. Each individual is assigned a number in sequence as it is removed from the parents' cage. There is no assurance that the order of assignment of numbers to the individuals of a litter is random, since persons assigning the numbers view the entire litter beforehand. It is known that in the case of visible segregation, some persons will pick out all individuals of one class to mark first; therefore, if the individual is to be used as the basis for sampling, it is necessary that the order of individuals within each litter be randomized.

Since it is sufficient, for termination of the experiment, to have the cumulative number of recombinants lie outside the decision lines only once, and since it is certain that in some cases the point will fluctuate across a line, it is desirable to use small groups or individuals as the final point approaches the line closely. To fail to do so may require the rearing of one or more additional groups. On the other hand, if the final point lies outside the decision lines, no matter how large the group, the experiment may be terminated at once.

DESCRIPTION OF GENES

Since most of the genes involved in these linkage tests have been described previously, only brief descriptions are repeated.

BROWN-TIP (b).—The gene was described by Huestis and Barto (1934) in the subspecies gambeli from Oregon. It was later reported by Blair (1947) to occur in blandus from New Mexico, and the two were shown to be identical. The name brown agouti was used by Huestis and Barto; the name brown-tip was proposed by Blair. The genes used in the present studies were derived from Blair's stocks. In the wild (black-tip) phenotype the tips and bases of the hairs, as well as the entire shafts of non-agouti hairs, are nearly black. A brown-tip mouse has hairs which are brownish in the same regions. Black-tip appears to be completely dominant, and segregation is according to expectation.

DILUTE (d).—The gene was described by Dice (1933) in blandus from New Mexico. Dilute mice are characterized by reduced pigmentation in the tips and bases of the hairs. Some hairs may be entirely devoid of black pigment. The character is variable in manifestation, ranging from easily recognizable animals to those which overlap the normal condition. Segregation for dilute, therefore, is usually, but not seriously, deficient. A useful microscopic character is the presence of clumps of pigment, particularly in the bases of the hairs. Such clumping will identify some dilute mice which cannot be so identified macroscopically.

HAIRLESS (hr).–The gene was reported by Sumner (1924). At an early age, generally before six weeks, a genetically hairless mouse begins to lose its hair. Many mice develop white hairs while in the process of becoming hairless. The mice then remain hairless, except for infrequent small patches of hairs. If young hairless mice are observed before weaning, it is possible to classify them for other pelage characters which show up in the juvenile pelage. The normal allele is completely dominant, and segregation is according to expectation.

IVORY (i).–The gene was discovered by Huestis (1938), who trapped a wild ivory mouse of the subspecies rubidus in Oregon. Ivory mice are very similar to albino mice, except that ivory mice have a slight amount of pigmentation in the hairs. The wild allele is completely dominant, and ivory segregates as expected.

WALTZING (v).–The gene was described by Dice (1935) in bairdi from Iowa, and further data were reported by Watson (1939). A whirling type of behavior is evoked by disturbance and possibly by other stimuli. The normal allele appears to be completely dominant, and segregation is reasonably close to expectation.

WIDE-BAND (Nb).–Clark (1938) reported two linkage tests involving wide-band, but because the wide-band locus had not been recognized at the time, he confused it with the gray-band locus. Dice (1933) had reported the occurrence of dichromatism in the race blandus, and showed that the yellowish pelage, which he called buff, was due to a single dominant gene. The recessive allele produced a gray pelage. Clark (1938) applied the gene symbols A^b to buff and A to gray, thinking they were at the agouti locus.

Blair (1947) showed that the buff-gray conditions in blandus are due to the presence or absence of yellow pigment in the agouti bands of the hairs and adopted the names and symbols yellow-band (G) and gray-band (g). Dice (1941) reported that the bright yellow now known to be characteristic of the wide-banded phenotype is widely distributed among individuals of the race nebrascensis from the sand hills of Nebraska, but he did not then name the condition nor did he report on its inheritance. Wide-band has since been shown (unpublished) at the Laboratory of Vertebrate Biology to be a simple dominant to the more common narrow-band phenotype. The chief difference between the two consists of a lengthening of the agouti bands and a concomitant reduction in the frequency of non-agouti hairs in the dominant phenotype. The frequency of the wide-band gene in mice from the sand hills is presently unknown, nor is it known to what extent other genes are involved in producing the characteristic bright yellow, except that the yellow-band gene (G) is of very high frequency.

Subsequent work has failed to discover the wide-band gene (Nb) in the stocks of blandus descended from Blair's animals and has also failed to find the gray-band (g) in the descendants of Dice's nebrascensis stocks. Clark clearly stated that buff deermice of the subspecies nebrascensis were the source of the buff genes he used in the linkage tests. It appears highly probable, therefore, that Clark's linkage test No. 6, yellow vs. buff, was a test between yellow (y) and wide-band (Nb), and that his test No. 7, buff vs. hairless, was a test between wide-band (Nb) and hairless (hr). In addition to the possible confusion of genes, the results reported by Clark were based on, respectively, 160 and 88 mice. The small numbers alone were considered sufficient justification to repeat the tests.

YELLOW (y).—The gene was first described by Sumner (1917), who found it in gambeli from California. The name "yellow" is a misnomer, inasmuch as the gene appears to have nothing to do with the color of the agouti band. The effect of the recessive allele in double dose is to reduce the frequency of non-agouti hairs almost to zero, and to considerably increase the length of agouti bands in banded hairs. This change makes the color of the bands, usually yellow, a prominent feature, and since this was one of the earliest mutant genes known in peromyscus, the name was appropriate at the time. The effect of the yellow gene on the hairs is very similar to the effect of the wide-band gene, but certain microscopic differences allow the two traits to be distinguished in most cases.

ANALYSIS

In the discussion which follows, independent assortment will be claimed for six of the eight tests. To avoid repetition, the simple statement that no linkage exists will be made. This should be understood to mean the following: The probability that linkage passed undetected, in the event that the recombinant fraction actually is .40 or lower, is equal to or less than .01.

The conventional analysis of χ^2 is made in all cases. In sequential analysis, no less than in the previous methods, it is important to demonstrate that poor single factor segregation does not affect the proportion of recombinants. In some tests, for various reasons, more data were collected than were needed for the sequential test. The additional data are useful for the χ^2's, and are in all cases utilized.

WIDE-BAND (Nb) vs. WALTZING (v).—A testcross was made in coupling phase. The analyses for each sex are given in Tables I and II. It is apparent that total segregation is not in accord with the hypothesis that no linkage exists. It is also apparent that the single factors are segregating as expected. Further examination of the data by sibships (Mather, 1951, sec. 7) revealed that the several sibships were homogeneous for the single factor segregations and for the observed proportion of recombinants. The estimated recombinant fraction for females is .1055 ± .0144; that for males is .1139 ± .0152. The estimates for the sexes cannot be shown to differ.

TABLE I

Wide-band (Nb) vs. Waltzing (v), Females Only

Nb V/nb v x nb v/nb v

Class	Number	Analysis of χ^2			
Nb V	214	Source	χ^2	Df	P
Nb v	22	Nb:nb segregation	.6352	1	.5 - .3
nb V	26	V:v segregation	1.3736	1	.3 - .2
nb v	193	Linkage	283.2549	1	<< .01
Total	455	Total	285.2637	3	

$\Theta = .1055 \pm .0144$

During the collection of data it was suspected that the recombinant fraction was decreasing, at least for females, as the mice became older. Since most of the original matings involved mice older than six months when their first litters were born, additional matings were made utilizing younger mice. The final results discount the possibility of any appreciable effect of parental age on the recombinant fraction.

TABLE II

Wide-band (Nb) vs. Waltzing (v), Males Only

Nb V/nb v x nb v/nb v

Class	Number	Analysis of χ^2			
Nb V	186	Source	χ^2	Df	P
Nb v	24	Nb:nb segregation	.8223	1	.5 - .3
nb V	26	V:v segregation	.5125	1	.5 - .3
nb v	203	Linkage	261.7790	1	<< .01
Total	439	Total	263.1138	3	

$\Theta = .1139 \pm .0152$

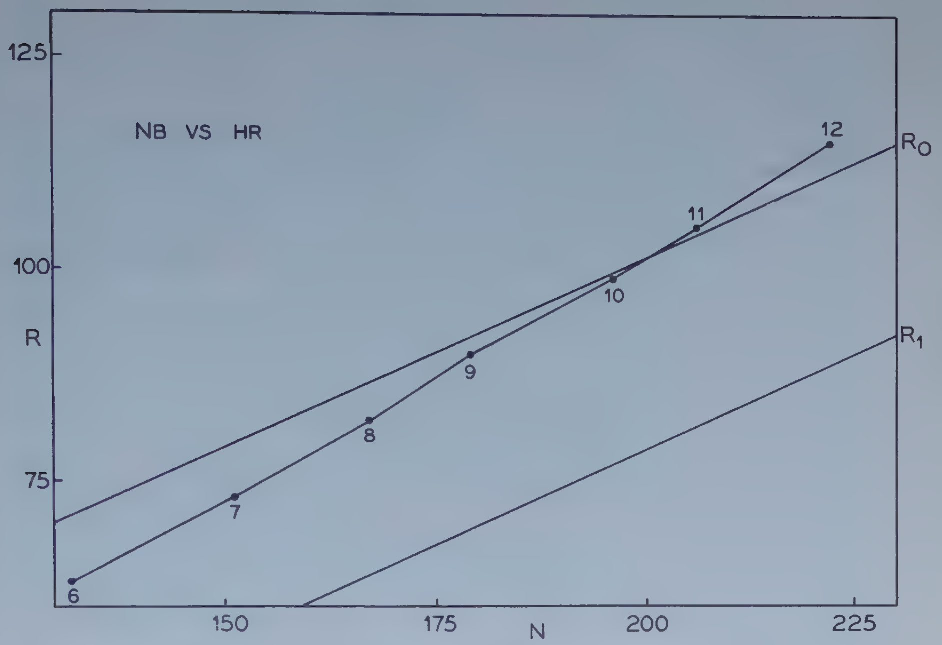

FIG. 2. Sequential test between wide-band and hairless, coupling testcross.

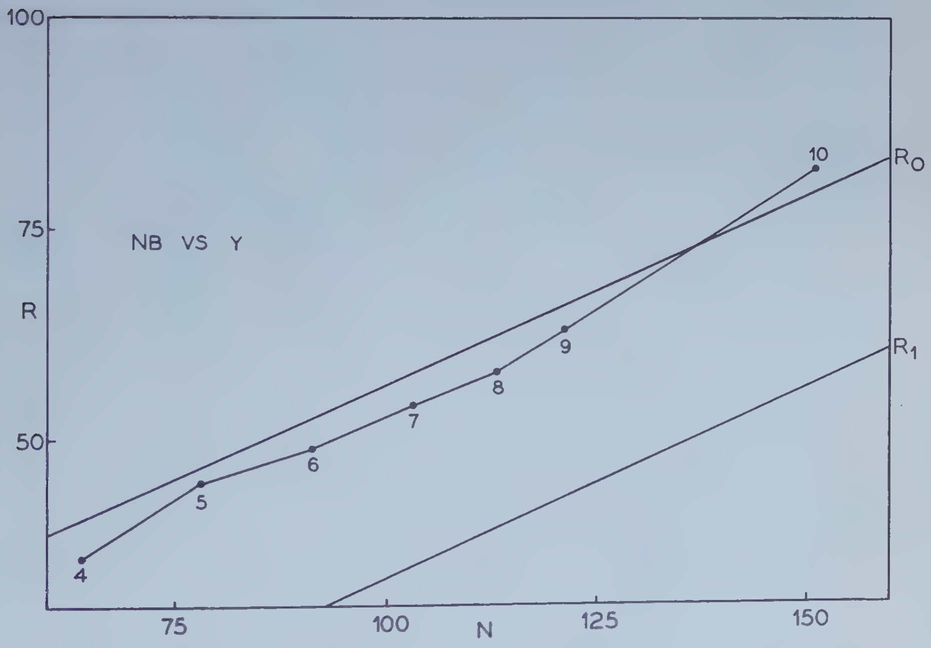

FIG. 3. Sequential test between wide-band and yellow, coupling testcross, epistasis.

WIDE-BAND (Nb) vs. HAIRLESS (hr).–A testcross was made in coupling phase. Data and analysis of χ^2 are given in Table III. The sequential analysis is shown in Figure 2. It will be noted that the experiment could have been terminated after the eleventh increment of data. In this test, because of the necessity of making the classification for hairless no earlier than about six weeks, the twelfth group had been marked before the eleventh group was classified. The additional group is useful in the analysis of χ^2, and is therefore included. There is no evidence for linkage between the wide-band and hairless loci.

WIDE-BAND (Nb) vs. YELLOW (y).–A testcross was made in repulsion phase. Yellow is partly epistatic to the wide-band locus. Classification is easily made in nonyellow mice, but not all yellow mice can be classified as wide-band or narrow-band. It is thus necessary to limit classification to three classes, respectively, wide-band nonyellow, narrow-band nonyellow, and yellow. This is an improvement over the classification used by Clark (1938) in making a test between the same loci. Clark was able to distinguish only the narrow-band nonyellow class and had to combine the other three possible classes. Certain microscopic hair characters not recognized by Clark enable wide-band nonyellow mice to be distinguished from yellow mice.

The data and analysis of χ^2 are given in Table IV. The sequential analysis is shown in Figure 3. This test requires additional consideration. Both the sequential test and the χ^2 for linkage in reality are functions of segregation for Nb-nb and segregation for recombinant-parental gametes. Many possible combinations of linkage and disturbed segregation would lead to the same χ^2 and sequential test obtained for the present test. In the absence of assurance that the Nb-nb segregation is within the usual limits of expectation, no statement about the presence or absence of linkage can be made. No internal evidence is available from the present linkage test to answer the question.

Other tests reported in this paper, however, give an indication of the segregation to be expected. Data from Tables I, II, and III show that of 1116 mice reared in those linkage tests, 566, or 50.7 per cent ($\chi^2 = .032$; $.9 > P > .8$), were wide-banded. In view of this evidence, it appears reasonable to accept the judgment of the sequential analysis and to state that there is no linkage between the wide-band and the yellow locus.

IVORY (i) vs. YELLOW (y).–A testcross was made in repulsion phase. Ivory is completely epistatic to the yellow locus and consequently only the classes nonivory nonyellow, nonivory yellow, and ivory can be distinguished. Data and analysis of χ^2 are given in Table V. The sequential analysis is shown in Figure 4. The collection of data could have been terminated after the ninth group was collected. This test was under way when it was decided to apply sequential analysis, thereby accounting for the failure to terminate at the indicated point. In view of the epistasis, however, and the consequent inability

TABLE III

Wide-band (Nb) vs. Hairless (hr)

Nb Hr/nb hr x nb hr/nb hr

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
Nb Hr	59				
Nb hr	61	Nb:nb segregation	1.4595	1	.3 - .2
nb Hr	54	Hr:hr segregation	.0721	1	.8 - .7
nb hr	48	Linkage	.2883	1	.7 - .5
Total	222	Total	1.8199	3	

to determine just what the Y-y segregation is in this particular test, the additional group certainly lends weight to the conclusion that no linkage exists.

Data from Tables IV and VII give some indication of the segregation to be expected at the y locus. Of 613 mice reared, 302, or 49.3 per cent (χ^2 = .132; .8 > P > .7), were nonyellow. It therefore appears reasonable to state that no linkage exists between the ivory and the yellow loci.

TABLE IV

Wide-band (Nb) vs. Yellow (y)

Nb y/nb Y x nb y/nb y

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
Nb Y	82				
nb Y	69	Y:y segregation	.0537	1	.9 - .8
y	147	Linkage	1.1342	1	.3 - .2
Total	298	Total	1.1879	2	

BROWN-TIP (b) vs. WALTZING (v).–A testcross was made in coupling phase. The data and analysis of χ^2 are given in Table VI, and the sequential analysis in Figure 5. There is no evidence of linkage.

TABLE V

Ivory (i) vs. Yellow (y)

I y/i Y x i y/i y

Class	Number	Analysis of χ^2			
I Y	123	Source	χ^2	Df	P
I y	106	I:i segregation	.0195	1	.9 - .8
i	232	Linkage	1.2538	1	.3 - .2
Total	461	Total	1.2733	2	

BROWN-TIP (b) vs. YELLOW (y).—A testcross was made in repulsion phase. The data and analysis of χ^2 are presented in Table VII, and the sequential analysis in Figure 6. This test had been completed before sequential analysis had been applied to any linkage tests. Since the data occurred in groups, just as for the other tests, they were reconstructed as an illustration of the savings which would have resulted by the application of sequential analysis. Figure 6 shows that the sixth group would have served to terminate the analysis, after 149 mice had been examined. Note also that the seventh group has dropped below the R_0 line and that the eighth and subsequent points again lie above the line. The experiment was terminated, according to some now forgotten criterion, after 315 testcross mice had been

TABLE VI

Brown-tip (b) vs. Waltzing (v)

B V/b v x b v/b v

Class	Number	Analysis of χ^2			
B V	39	Source	χ^2	Df	P
B v	46	B:b segregation	.2045	1	.7 - .5
b V	50	V:v segregation	.0227	1	.9 - .8
b v	41	Linkage	1.4545	1	.3 - .2
Total	176	Total	1.6817	3	

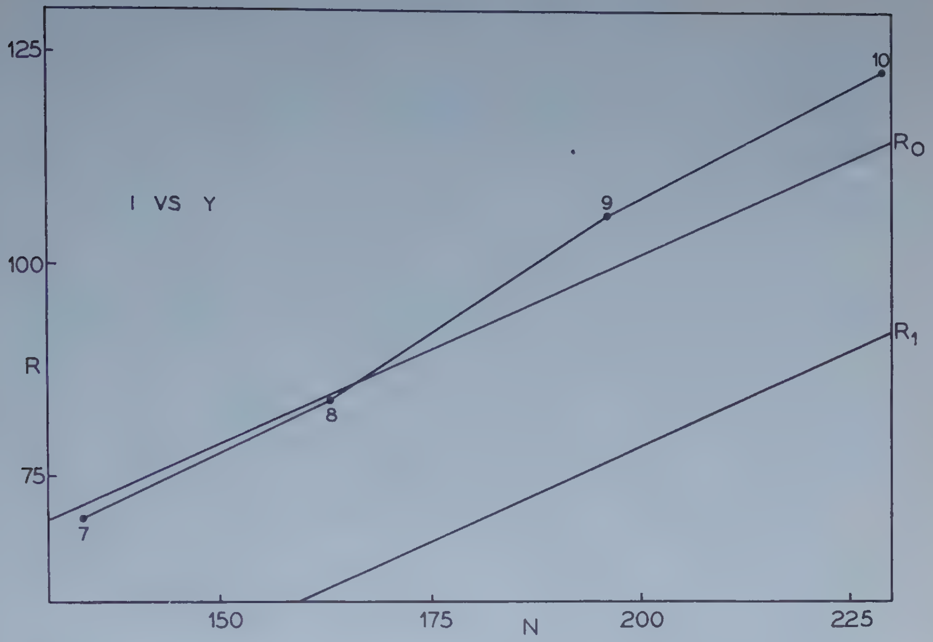

FIG. 4. Sequential test between ivory and yellow, repulsion testcross, epistasis.

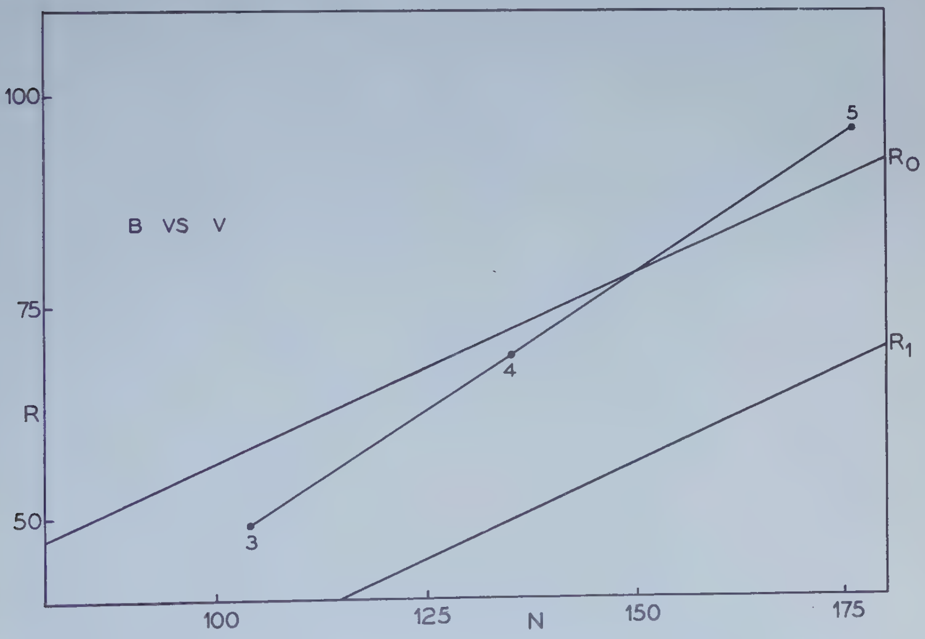

FIG. 5. Sequential test between brown-tip and waltzing, coupling testcross.

reared and classified. The test could have been terminated after only 47 per cent of the total mice were collected. The saving of 166 testcross progeny and the labor expended on collecting the information might well have been enough to complete another test. The average sample number (equation 5) for the test used is 221; the other four testcrosses in which independent assortment was demonstrated could have been terminated with, respectively, 151, 176, 196, and 206 usable mice. The conclusion, however, is completely established that no linkage exists between the brown-tip and the yellow loci.

TABLE VII

Brown-tip (b) vs. Yellow (y)

B y/b Y x b y/b y

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
B Y	84				
B y	85	B:b segregation	1.6794	1	.2 - .1
b Y	67	Y:y segregation	.5365	1	.5 - .3
b y	79	Linkage	.3841	1	.7 - .5
Total	315	Total	2.6000	3	

TABLE VIII

Brown-tip (b) vs. Hairless (hr)

B hr/b Hr x B hr/b Hr

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
B Hr	407				
B hr	118	B:b segregation	.2775	1	.7 - .5
b Hr	130	Hr:hr segregation	2.4971	1	.2 - .1
b hr	37	Linkage	.0026	1	.98 - .95
Total	692	Total	2.7772	3	

BROWN-TIP (b) vs. HAIRLESS (hr).—A repulsion F_2 generation is the basis for the test. The data and the analysis of χ^2 are given in Table VIII. The sequential analysis is shown in Figure 7. The test could have been terminated after the third marking group. The fourth

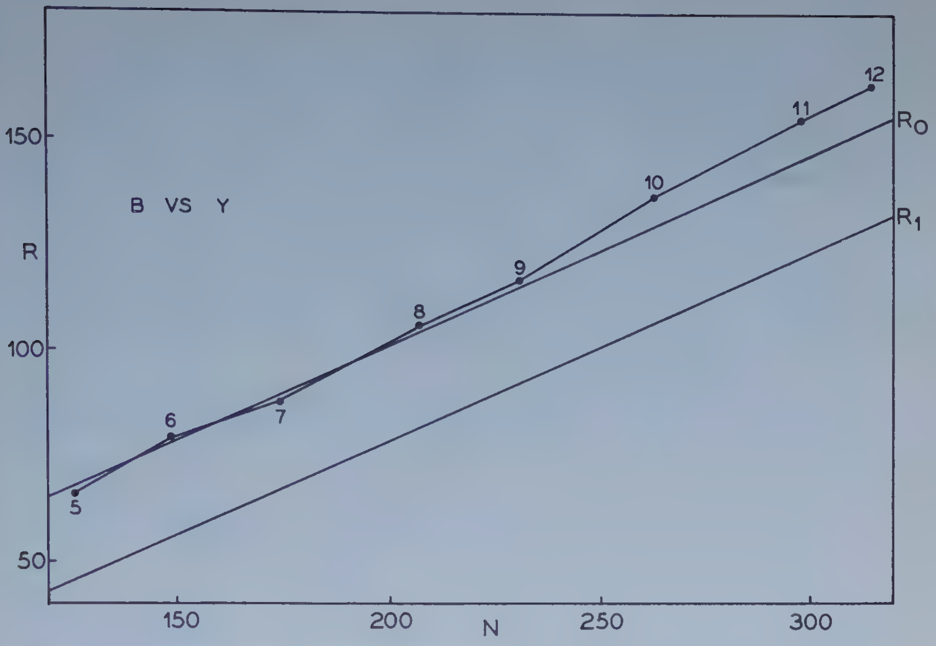

FIG. 6. Sequential test between brown-tip and yellow, repulsion testcross.

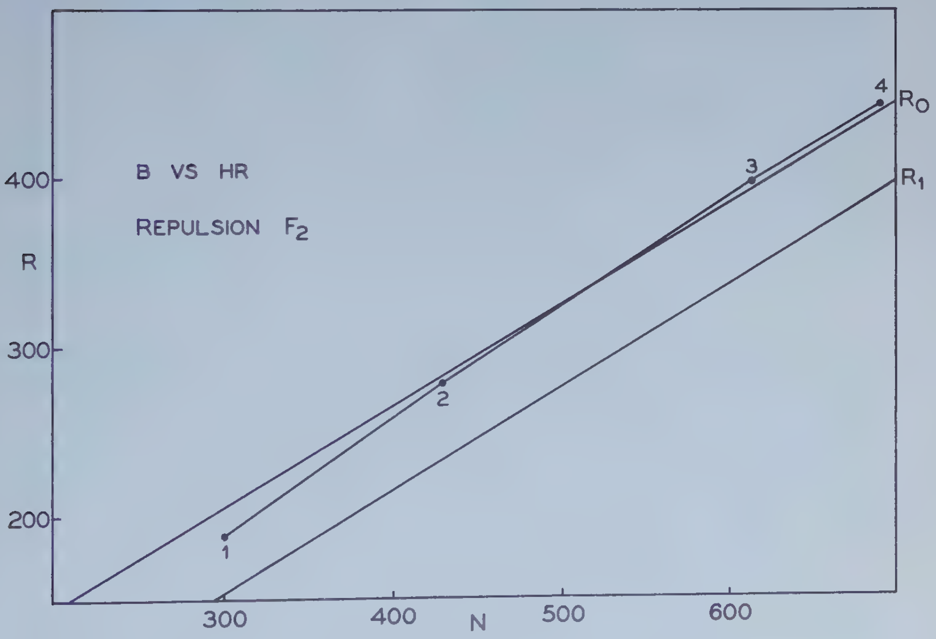

FIG. 7. Sequential test between brown-tip and hairless, repulsion F_2.

group was collected while the third group was being held until six weeks of age so that hairless could be classified. There is no evidence of linkage between the brown-tip and the hairless loci.

BROWN-TIP (b) vs. DILUTE (d).—A testcross was made in coupling phase. Brown-tip is epistatic to dilute, insofar as macroscopic classification is concerned. Microscopically, the clumping of pigment which is characteristic of dilute mice is present in brown-tip dilute mice, but to a lesser extent than in black-tip dilute ones. The segregation ratios obtained for D-d would indicate that the proper classification can be made microscopically. Some difficulties were experienced with the test, however, which possibly were due to improper classification.

Detection and estimation of linkage for the male F_1's were without complications. The data and analysis of χ^2 are presented in Table IX.

TABLE IX

Brown-tip (b) vs. Dilute (d), Males Only

B D/b d x b d/b d

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
B D	54				
B d	47	B:b segregation	.0452	1	.9 - .8
b D	32	D:d segregation	3.6633	1	.1 - .05
b d	66	Linkage	8.4472	1	< .01
Total	199	Total	12.1557	3	

$\Theta = .3970 \pm .0347$

The group as a whole shows a shortage of recombinant gametes, and the D-d segregation is on the border of suspicion, with an excess of dilute mice. Fifteen males fathered the 199 testcross progeny, and these males belong to three sibships. An analysis for heterogeneity with respect to the proportion of recombinants produced by the males and by the male sibships was made, following Mather (1951, sec. 7). The results are given in Table X.

The χ^2's show that no heterogeneity is apparent either among individual males when corrected for their sibship totals or among the sibships of males. All agree in showing a shortage of recombinant gametes. Therefore, the estimated recombination fraction of .3970 ± .0347 for the males appears to be valid.

For the females there are some disturbing factors. Twenty-two females left offspring. Of these, one female produced 18 nondilute and no dilute young. A second female, a sib of the first, produced 33 nondilute offspring and only four dilute ones. For some undetermined reason these females were not segregating properly for dilute, and the two progenies were eliminated from the analysis. For the remaining 20 females the results shown in Table XI were obtained.

From Table XI everything appears to be in order; however, when an analysis is made among the females with respect to the proportion of recombinant offspring produced, as was made for the males, a distinct heterogeneity is apparent. These 20 females belong to four sibships, segregation data for which are given in Table XII.

TABLE X

Analysis of χ^2 for Heterogeneity Among Male F_1's, Brown-tip vs. Dilute

Source	χ^2	Df	P
Deviation from 1:1 ratio	8.4472	1	< .01
Among male sibships	1.3522	2	.7 - .5
Among individual males	11.9508	12	.5 - .3
Total	21.7502	15	

TABLE XI

Brown-tip (b) vs. Dilute (d), Females Only

B D/b d x b d/b d

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
B D	109	B:b segregation	.0457	1	.9 - .8
B d	68	D:d segregation	.0114	1	.95 - .9
b D	67	Linkage	18.2857	1	<< .01
b d	106				
Total	350	Total	18.3428	3	

Analysis of χ^2 for these sibships shows that heterogeneity is present among sibships; the χ^2 is 15.610, with three degrees of freedom and a probability of less than .01. Inspection points to sibship A as probably being the cause of the heterogeneity. This sibship consists of four females, whose segregation is shown in Table XIII.

Analysis of χ^2 within this sibship again indicated heterogeneity for proportion of recombinants. The χ^2 is 12.335, with three degrees of freedom and a probability of less than .01. This is the sibship to which the previously eliminated mice belong.

TABLE XII

Segregation of Recombinant Gametes by Sibship

of the Mothers, Brown-tip vs. Dilute

Sibship	Recombinant gametes	Total gametes	Θ
A	13	67	.194
B	37	100	.370
C	9	22	.409
D	76	161	.472

No explanation is presently available for the behavior of this one group of mice. One possibility, the poor dilute segregation, needs investigation. Three strains which have a very similar phenotype are maintained in the laboratory. All are recessives. Possibly one of the other genes is mixed in what was thought to be a pure dilute strain.

TABLE XIII

Segregation of Recombinant Gametes from Females

Belonging to One Sibship, Brown-tip vs. Dilute

Female	Recombinant gametes	Total gametes	Θ
1	3	14	.214
2	1	24	.042
3	7	14	.500
4	2	15	.133
Total	13	67	.194

A possible explanation for the heterogeneity of recombinant segregation is that some individuals may carry an inversion in the appropriate chromosome. Pending answers to the questions, an analysis was made for females after elimination of all testcross progeny resulting from this questionable sibship. The analysis for the remaining mice is shown in Table XIV.

TABLE XIV

Brown-tip (b) vs. Dilute (d), Females Only, Certain Females Eliminated

B D/b d x b d/b d

Class	Number	Analysis of χ^2			
		Source	χ^2	Df	P
B D	76	B:b segregation	.5972	1	.5 - .3
B d	59	D:d segregation	.0883	1	.8 - .7
b D	63	Linkage	5.3746	1	.05 - .02
b d	85				
Total	283	Total	6.0601	3	

$\Theta = .4311 \pm .0294$

The group as a whole still shows a shortage of recombinants. A final analysis of heterogeneity gives the results shown in Table XV. The group now appears to be homogeneous. The recombination fraction for females is estimated to be .4311 ± .0294. The group eliminated from the final analysis possessed a lower recombination

TABLE XV

Analysis of χ^2 for Heterogeneity Among Female F_1's, Certain Females Omitted, Brown-tip vs. Dilute

Source	χ^2	Df	P
Deviation from 1:1 ratio	5.3746	1	.05 - .02
Among female sibships	2.6667	2	.3 - .2
Among individual females	7.5200	13	.9 - .8
Total	15.5613	16	

fraction; accordingly, if any bias is thereby introduced, it is on the conservative side.

I have some hesitance in reporting the results of this test since they are unsatisfactory. In practice, however, since brown-tip and dilute are reported to be so loosely linked, tests of other loci for independent segregation against the present linkage group would have to be made against both dilute and brown-tip. Hence it does not appear that a report of probable linkage between these two genes would prevent others from making any linkage tests which might otherwise be performed.

DISCUSSION

Use of sequential analysis for linkage tests in peromyscus is seen to result in:

a. A precise specification of the alternate hypotheses.

b. A predetermination of error levels for both types of errors which may be made in a sampling process.

c. Attainment of the desired results with the minimum possible sample size, a considerable saving on the average over the conventional method. The usual method, furthermore, generally specifies only one alternative and usually specifies the level for the type I error only.

Sequential tests are not useful in the cases in which linkage does exist, except for detection, but they involve no wastage of effort. Estimation must be made by conventional means. Sequential tests are most useful for detection of independent assortment, where estimation is meaningless, and such tests eliminate the vagueness sometimes associated with reports of cases of independent assortment.

The present data place one, and probably two, additional peromyscus genes into linkage groups, but are insufficient to establish with reasonable probability any additional marker genes.

The most recent summary of the chromosome map of peromyscus was made by Barto (1942). At that time linkage group I contained albino (c) and pinkeye (p), group II contained yellow (y), group III contained hairless (hr), and group IV contained dilute (d). Since then, two additional genes, flexed tail (f) and silver (sl), have been placed in linkage group I; flexed tail is linked with silver (Huestis and Piestrak, 1942), and with albino (Huestis and Lindstedt, 1946). The present data probably add brown-tip (b) to linkage group IV and show that waltzing (v) and wide-band (Nb) are closely linked.

Discovery of linkage between flexed and albino, with roughly 35 per cent crossing over, makes additional tests necessary to establish independent assortment of other marker genes from those of group I. It would be desirable if the linear order of the group I genes were known, but in the absence of such information, it is still known that the first chromosome is long enough so that tests against one marker alone will not serve to establish independent assortment. Linkage groups II, III, and IV had all been established previously by tests against albino alone, and thus none of those groups can now be considered to be completely tested against group I.

Barto (1942) reported that waltzing segregates independently of albino. Clark (1938) and the present report show that wide-band segregates independently of the known markers for groups II and III. The present paper demonstrates independent segregation of waltzing against brown-tip of group IV. It is thus possible that waltzing and wide-band may represent markers for a fifth linkage group in peromyscus. To complete the establishment of this group, tests are needed between wide-band or waltzing and flexed-tail of group I, and with dilute of group IV. If such tests show independent segregation, then group V could be considered established insofar as the presently known markers will permit.

The possible status of ivory as a marker is still uncertain. It has now been shown to segregate independently of groups II, III, and the dilute locus of group IV. Tests are still needed against the brown-tip locus of group IV, and against group I and the tentative group V before ivory may stand as a probable marker for a sixth linkage group.

Additional linkage tests have been reported heretofore, but they deal with other genes, and do not at present contribute appreciably to the establishment of additional marker genes.

SUMMARY

Sequential tests have been applied to linkage tests in peromyscus. Their use enables any desired degree of precision to be obtained with the minimum possible effort in each case, and the final answers are less vague than those obtained by the conventional method.

The following pairs of loci are shown to segregate independently: wide-band (Nb) and hairless (hr), wide-band and yellow (y), yellow and ivory (i), brown-tip (b) and waltzing (v), brown-tip and yellow, and brown-tip and hairless.

Wide-band and waltzing are linked with a recombination fraction of .1055 ± .0144 for females and .1139 ± .0152 for males. Brown-tip and dilute (d) are probably linked with a recombination fraction of .4311 ± .0294 for females and .3970 ± .0347 for males. The recombinant fraction for females may be biased toward .50 because of the elimination of some questionable data which showed a lower recombinant fraction.

The wide-band and waltzing loci may tentatively be considered to represent a fifth linkage group in peromyscus.

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